The Salt Stress-Inducible Protein Kinase Gene, *Esi47*, from the Salt-Tolerant Wheatgrass *Lophopyrum elongatum* Is Involved in Plant Hormone Signaling[1]

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Protein kinases play a central role in signal transduction in all organisms and to study signal transduction in response to salt stress we have identified and characterized a gene encoding a protein kinase that is induced by salt stress and abscisic acid (ABA) in the salt-tolerant wild wheatgrass *Lophopyrum elongatum* (Host) A. Love. The product of the early salt stress-induced gene, *Esi47*, was found to belong to the "novel Arabidopsis protein kinase" group of plant serine/threonine protein kinases. Transient gene expression assays in barley aleurone tissue showed *Esi47* to suppress the gibberellin induction of the barley low-pI α-amylase gene promoter, thus providing evidence for the role of this protein kinase gene in plant hormone signaling. *Esi47* contains a small upstream open reading frame in the 5′-untranslated region of its transcript that is implicated in mediating the repression of the basal level of the gene expression and in regulating the ABA inducibility of the gene, as shown in the transient gene expression assay in maize callus. Three Arabidopsis homologs of *Esi47* were identified, and different members of this clade of genes showed differential patterns of regulation by salt stress and ABA in Arabidopsis roots and leaves. At least one of the Arabidopsis homologs contains a small open reading frame in its 5′-untranslated region, indicating that the unusual regulatory mechanism identified in *Esi47* may be widely conserved.

Grass species include the most important crop plants for world food production. They also have a rich degree of genetic variation for adaptation to extreme environmental stress conditions, including high salinity, high and low temperatures, and drought. This genetic variation is likely the cause and the result of their importance as crop species. As cereals were spread from their centers of origin by trade and human migration, they came to be adapted to cultivation in a wide range of climatic conditions. A genetic basis for tolerance to extreme environmental stress is still more striking among the wild relatives of the cultivated cereals. They are a valuable resource in studying the basis of stress tolerance and the advent of transgenic methodologies make these species a rich potential source of genes for the improvement of crop species. The impact on environmental stress tolerance of the collective genetic model of the grasses will likely be significant within the Triticeae, including wheat, barley, rye, and related wild relatives. These species share a high degree of DNA sequence similarity and conservation of genome structure and gene order (Devos and Gale, 1997, 2000) and they have a high degree of genetic variation for tolerance to environmental stresses (McGuire and Dvořák, 1981; Ellis et al., 2000).

Several genes have been identified that are induced in the roots of the wild wheatgrass *Lophopyrum elongatum* (Host) A. Love (syn. *Elytrigia elongata* [Host] Nevski, *Agropyrum elongatum* Host) within 6 h of the commencement of salt stress and have been designated as early salt stress-inducible genes (*Esi*; Gulick and Dvořák, 1990, 1992). In this study we report the characterization of *Esi47*, which encodes a protein Ser/Thr kinase. *L. elongatum* is a highly salt-tolerant species that occurs naturally in salt marshes of the Mediterranean region (McGuire and Dvořák, 1981). This diploid species ($2n = 2x = 14$) has a close phylogenetic relationship to cultivated bread wheat and gene mapping to the level of the chromosome arm indicates colinearity of the two genomes (Dubcovsky et al., 1994). *L. elongatum* has been hybridized to salt-sensitive wheat and the octaploid amphiploid derived from the cross is able to grow, flower, and set seed when it is grown in 250 mM NaCl (Dvořák and Ross, 1986).

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Salt stress poses two primary challenges to a plant: ion toxicity and lowered water potential (Serrano and Gaxiola, 1994). The aspect of diminished water potential is shared with other environmental stresses, namely drought and low temperature stress (Bohnert et al., 1995; Ingram and Bartels, 1996; Bray, 1997). The altered level of expression of many genes is thought to be responsible for stress adaptation and tolerance. A number of genes that are induced by salt stress have been identified and many of these have also been found to be induced by drought and low temperature (Bohnert et al., 1995; Ingram and Bartels, 1996; Bray, 1997; Shinozaki and Yamaguchi-Shinozaki, 1997). Mechanisms of tolerance vary in expression between species and between tissues at different stages of development (Colmer et al., 1995). The complexity of the genetic basis of salt tolerance underscores the importance of the signal transduction mechanisms by which plants perceive and respond to salt stress. The understanding of the signal transduction pathways of the stress response will likely lead to the identification of key components that regulate the genetic response of the plant in a global manner.

The plant hormone abscisic acid (ABA) is an important intermediate in transducing signals of osmotic stresses since the ABA levels are often elevated upon stress and most, although not all, of the stress-responsive genes are also induced by external ABA treatment (Skriver and Mundy, 1990; Bray, 1997; Shinozaki and Yamaguchi-Shinozaki, 1997; Leung and Giraudat, 1998). ABA-insensitive mutants, *abi*, display a range of phenotypes including reduced seed dormancy, wilted seedlings, increased sensitivity to desiccation, and diminished induction of normally ABA-inducible genes. That *ABI1* and *ABI2* encode protein phosphatases (Leung et al., 1994, 1997; Meyer et al., 1994) indicates that protein phosphorylation plays an important role in ABA signaling pathways and is likely to be important in other stress responsive pathways. Protein kinases are recognized as having a central role in the control of signal transduction and a number of stress- or ABA-regulated protein kinase genes have been implicated in stress and ABA signaling in plants. The Ca^{2+}/calmodulin-dependent protein kinase genes from Arabidopsis, *ATCDPK1* and *ATCDPK1a*, activate the promoter of the barley stress-inducible gene *Hva1* when transiently expressed in maize leaf protoplasts (Sheen, 1996). Mutations in the Arabidopsis SNF1-like protein kinase gene *SOS2* renders plants hypersensitive to salt stress; the *SOS2* gene is able to activate a gene for plasma membrane Na^+/H^+ antiporter probably in a Ca^{2+}-dependent manner (Halfter et al., 2000; Liu et al., 2000; Shi et al., 2000). The wheat stress- and ABA-inducible gene *PKABA1* also encodes an SNF1-like protein kinase and has been shown to mediate ABA-promoted suppression of gene induction by gibberellin (GA; Gómez-Cadenas et al., 1999). The number and types of stress- and ABA-regulated plant protein kinase genes identified has grown substantially with the completion of the Arabidopsis genome sequencing. This allows us to investigate any group or subgroup of kinases in the aspects of their regulation and their involvement in stress and ABA signaling.

In this paper we report the identification of the protein kinase gene *Esi47* from the wheatgrass *L. elongatum*. It is most similar to a group of Ser/Thr protein kinases designated only as novel Arabidopsis protein kinases (NAKs; Moran and Walker, 1993; Hardie, 1999) for which no putative functions or patterns of expression have been described. *Esi47* was previously described as a partial cDNA clone and was shown to be induced in the roots *of L. elongatum* by NaCl treatment and to a lesser extent by ABA treatment (Galvez et al., 1993). In comparisons among *L. elongatum*, wheat, and their amphiploid, the level of induction of the gene correlated with the level of salt tolerance of the species (Galvez et al., 1993). To study this stress-regulated protein kinase we have isolated full-length cDNA and genomic DNA for *Esi47* and have assessed its involvement in plant hormone signaling. *Esi47* is shown to suppress the GA activation of gene expression in barley aleurone and, therefore, might be involved in the same signaling pathway as that for *PKABA1* (Gómez-Cadenas et al., 1999). In addition, the ABA regulation of *Esi47* itself involves elements in the 5′-untranslated region (UTR) of its transcript. We have also identified the Arabidopsis homologs of *Esi47* and we demonstrate that salt stress and ABA differentially regulate them.

RESULTS

Esi47 Encodes a Plant NAK Group Protein Ser/Thr Kinase

The original *Esi47* cDNA clone isolated from the subtractively enriched cDNA library had an insert size of 810 bp, whereas the northern-blot hybridization analysis revealed an mRNA band of about 1.7 kb (Gulick and Dvořák, 1990, 1992). This cDNA insert was used as probe to isolate full-length cDNA clones for the *Esi47* gene from a cDNA library derived from mRNAs of *L. elongatum* roots treated with 250 mM NaCl for 6 h. Among the positive clones selected, the longest cDNA insert was 1.8 kb in length and contained a major open reading frame (ORF) of 1,299 bp, which translated into a protein of 433 amino acid residues with a molecular mass of 49,380 D. The deduced amino acid sequence of the gene product shows similarity to plant protein Ser/Thr kinases and contains a catalytic domain of 281 amino acid residues (Fig. 1). The amino and carboxyl terminal non-catalytic domains have 89 and

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MQCFRFASWEKEREEELQGPARSQSALSNS   30
SMSTDRDARRSGSECCSLTVSSEISVDSFG   60
RYRQLSLPHRPNNDLRIFTFQELKSATRSF   90
SRALMIGEGGFGCVYRGTIQSTLEPRRSLD  120
VAIKQLGRKGLQGHKEWVTEVNFLGVVDHP  150
NLVKLIGYCAEDDERGIQLLLVYEFMPHGS  180
LADHLSTRSPKPASWAMRLRVALDTARGLK  210
YLHEDSEFKIIFRDLKPSNILLDENWNAKL  240
SDFGLARLGPQEGSHVSTAVVGTIGYAAPE  270
YIHTGRLSSKNDIWSYGVVLYELLTGRRPL  300
DRNRPRGEQNLVEWVKPYSSDTKKFETIMD  330
PRLEGNYNLKSAARIASLANKCLVRHARYR  360
PKMSEVLEMVQKIVDSSDLGTPERPLISHS  390
KKLASDEKKRKGLNLKRRIADIKAGDGRWF  420
RWHKWTPKLVRTQ                   433
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Figure 1. *Esi47* encodes a protein Ser/Thr kinase-like protein. The amino acid sequence is deduced from the nucleotide sequence of the *Esi47* cDNA (GenBank accession no. AF131222). The catalytic domain is underlined. The amino acid residues conserved in all protein Ser/Thr kinases and protein Tyr kinases (Hanks and Quinn, 1991) are shown in bold letters; the positions at which the *Esi47* gene product has an amino acid residue other than the one conserved in protein Tyr kinases (Hanks and Quinn, 1991) are highlighted.

63 amino acid residues, respectively (Fig. 1). The catalytic domain of Esi47 contains all the invariant amino acid residues conserved in all protein Ser/Thr kinases; in contrast, it does not have seven of the residues conserved in protein Tyr kinases (Fig. 1; Hanks et al., 1988; Hanks and Quinn, 1991).

A comparison of the *Esi47* amino acid sequence with those of the proteins in the sequence databases showed that the Esi47 protein is most similar to three putative protein Ser/Thr kinases predicted from the Arabidopsis genome sequence. These Arabidopsis kinases, F8A24.12, F12E4.50, and T7F6.28, have 59%, 59%, and 54% overall amino acid sequence identity with Esi47, respectively, and have 70%, 70%, and 68% identity, respectively, within the catalytic domain. The most similar plant protein kinase to Esi47 for which some functional description is available is the Arabidopsis protein APK1 (Hirayama and Oka, 1992), which shows 45% and 54% sequence identity in whole protein and in catalytic domain to Esi47, respectively. Furthermore, 10 proteins most similar to Esi47 were retrieved from the protein databases by BLASTP search. These kinases and representatives from several major groups of plant protein kinases were used to build a phylogenetic tree (Fig. 2). The tree clearly shows that Esi47, along with F8A24.12, F12E4.50, T7F6.28, and APK1, belong to the NAK group of plant protein Ser/Thr kinases (Hardie, 1999). Esi47, F8A24.12, F12E4.50, T7F6.28, and another Arabidopsis putative protein kinase, T9I4.2, cluster in a clade other than the one that includes APK1 and NAK (Fig. 2). Therefore, Esi47 represents a unique subgroup in the NAK group of plant protein kinases.

The *Esi47* Gene Contains a Short Upstream ORF and a 5′-UTR Intron

Genomic clones for *Esi47* were isolated from an *L. elongatum* genomic DNA library. The sequence of the *Esi47* genomic DNA was determined from 3 kb upstream to 0.5 kb downstream of the protein kinase-coding region. The transcription initiation site was determined by primer extension with an antisense primer 64 bp downstream of the first nucleotide of the longest cDNA insert (Fig. 3). The extension product was 75 bp. Therefore, the longest cDNA insert obtained only lacks 11 bp of the transcribed sequence at the 5′ end (Fig. 3). Alignment of the cDNA and the genomic sequences of the *Esi47* gene revealed that the gene has four introns. The first intron is 312 bp in length and is located in the 5′-UTR (Figs. 3 and 4). The other introns are in the coding region and their lengths are 171, 142, and 602 bp, respectively (Fig. 4, sequence not shown). The DNA sequence in the exons is identical to the cDNA sequence. In addition, *Esi47* contains a short ORF of 51 bp upstream of the 5′-UTR intron and the protein kinase ORF (Figs. 3 and 4). This upstream ORF (uORF) can be translated into a sequence of 17 amino acid residues that has no

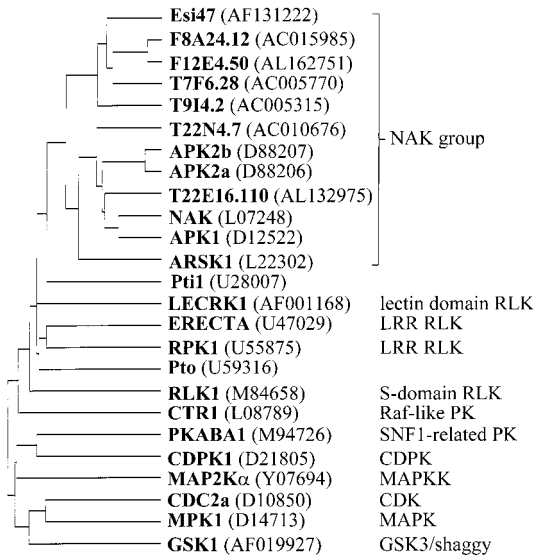

Figure 2. Esi47 belongs to a unique subgroup of the plant NAK group protein kinases. The amino acid sequences of the catalytic domains of the protein kinases shown were compared by the CLUSTAL method (Higgins and Sharp, 1988). The kinase groups, according to Hardie (1999), are indicated in the right column. The kinases in the NAK group, except for ARSK1, which are the most similar proteins to Esi47, were determined by BLASTP search in the databases. The database accession numbers for the genes encoding the kinases are shown in brackets. The two disease-resistance related protein kinases, Pto (Martin et al., 1993) and Pti1 (Zhou et al., 1995), are from tomato; the SNF1-related protein kinase PKABA1 (Anderberg and Walker-Simmons, 1992) is from wheat; the rest, except for Esi47, are from Arabidopsis. RLK, Receptor-like kinase; LRR, Leu-rich repeat; PK, protein kinase, CDPK, Ca^{2+}/calmodulin-dependent kinase; MAPK, mitogen-activated protein kinase; MAPKK, mitogen-activated protein kinase kinase.

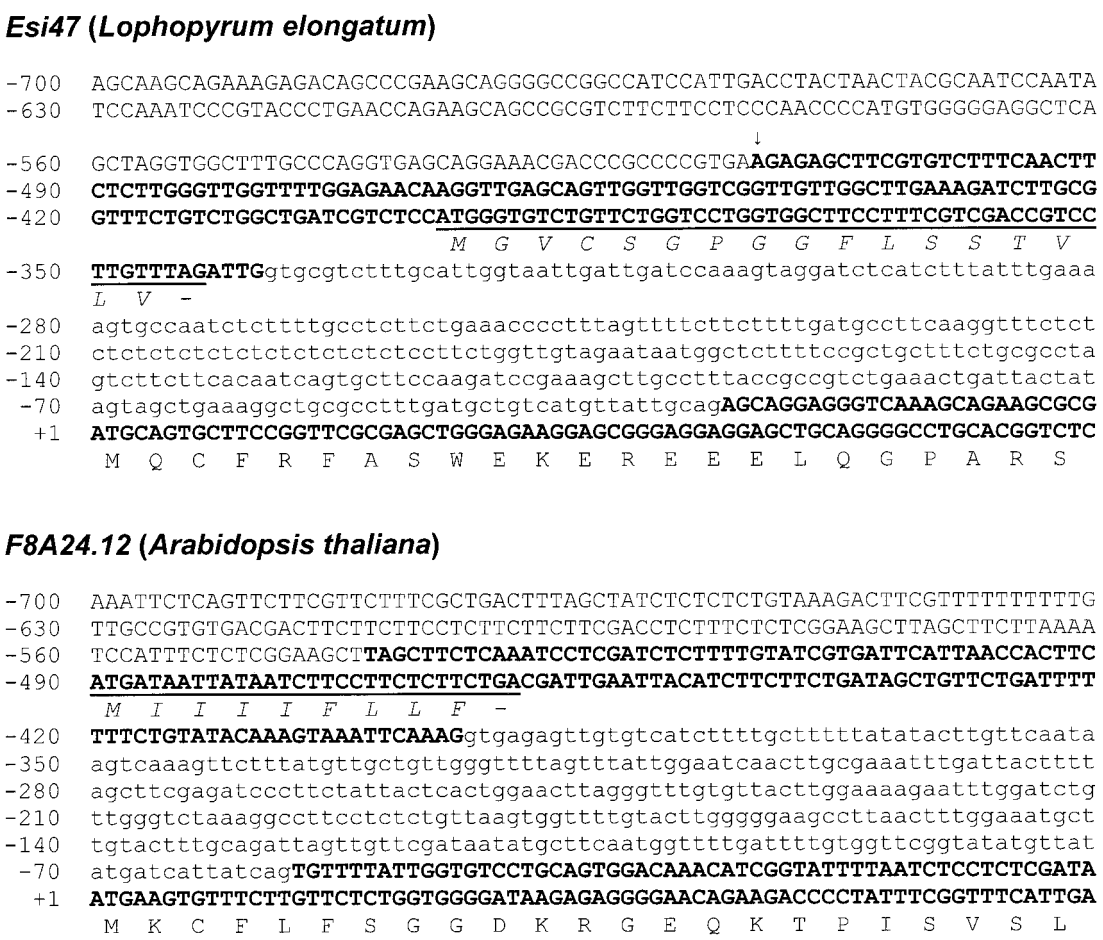

Figure 3. *Esi47* and its Arabidopsis homologous gene *F8A24.12* are similar in gene structure. The genomic DNA sequences corresponding to the 5′-UTRs of the two genes are shown. The bold letters indicate mRNA transcript region sequences confirmed by cDNA clones or the primer extension result. The transcription initiation site for *Esi47* determined by primer extension is indicated by the arrow. The 5′-UTR introns are shown in lowercase letters. The uORFs are underlined and the peptides they might encode are shown in italic letters below. The amino terminal amino acid sequences encoded by the major protein kinase ORFs are also shown. The first nucleotide of the kinase coding regions are designated positions +1. The GenBank accession numbers are AF339747 (*Esi47* genomic sequence), AC015985 (*F8A24.12* genomic sequence), and AI993614 (cDNA sequence for *F8A24.12* derived from the EST clone 701496785).

similarity to any of the protein sequences in the GenBank database (Fig. 3). The uORF and the major protein kinase ORF are separated by 33 nucleotides in the *Esi47* mRNA.

Esi47 Represses the GA Response in Barley Aleurone

To assess if *Esi47* has any regulatory activity in plant hormone signaling, barley aleurone was used as host for transient gene expression assay with particle bombardment for DNA delivery. As a control a mutant form of Esi47 was engineered in which the Lys-124 residue was changed to Gln (K124Q) to abolish the kinase activity since the Lys residue at this position is critical for the phosphate transfer by protein kinase (Hanks et al., 1988). The *Esi47* protein kinase-coding region or its K124Q mutant form was placed under the control of the strong and constitutively active promoter and the 5′-UTR intron of the rice actin gene, *Act1* (McElroy et al., 1990), to form the effector gene constructs *Act1-Esi47* or *Act1-Esi47*^{K124Q}. The influence of *Esi47* on the activities of the promoters of hormone-regulated genes fused to the reporter gene for β-glucuronidase (GUS) could thus be measured. The barley ABA-inducible *Hva1* gene promoter (Straub et al., 1994) and the barley GA-inducible low-pI α-amylase gene promoter were the two promoters tested in the forms of DNA constructs *Hva1-GUS* and *Amy-GUS*, respectively.

The *Hva1-GUS* chimeric gene was responsive to ABA in barley aleurone since the GUS activity was induced 27-fold by treatment with 20 μM ABA compared with the untreated tissues (Fig. 5). Cobombardment of *Hva1-GUS* with *Act1-Esi47* did not show any significant effects on the *Hva1* promoter when the aleurone tissues were not treated with ABA. Moreover, in the presence of *Act1-Esi47* the induction of *Hva1-GUS* by ABA was 28-fold, which was very

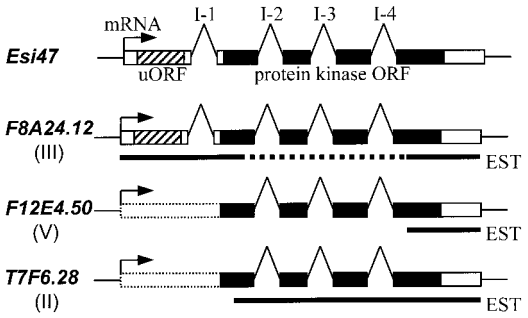

Figure 4. Schematic diagrams of the structures of *Esi47* and its Arabidopsis homologous genes. □, Untranslated exons; ■, protein kinase ORFs; ▨, uORFs; boxes with dotted lines, hypothetical 5′-UTRs for which no cDNA sequence is available; inverted "V," introns. For the EST clone representation, solid thick lines represent sequenced parts of cDNA insert of EST clone; dashed thick lines represent unsequenced parts of cDNA. The chromosomes on which the Arabidopsis genes are located are indicated in brackets. The introns of *Esi47* are numbered as I-1, I-2, I-3, and I-4. Introns of the Arabidopsis genes shown in the region not confirmed by cDNA sequences are predicted by gene annotation as provided in the GenBank entries. The regions are not drawn to scale.

similar to the induction rate in the absence of *Act1-Esi47* (Fig. 5). However, when the GUS values are compared, *Act1-Esi47* caused a minor, though statistically significant ($P = 0.014$), 24% reduction of the GUS activity in the ABA-treated aleurone. *Act1-Esi47*^{K124Q} had no effect on the *Hva1* promoter activity or the ABA induction of the *Hva1* gene (Fig. 5). This mutant form of *Esi47* also showed no significant difference from the wild-type *Esi47* gene in the GUS activities controlled by the *Hva1* promoter, regardless of ABA treatment (Fig. 5).

The GUS activity controlled by the barley α-amylase gene promoter could be induced 55-fold by treatment of 1 μM of GA when the barley aleurone tissues were bombarded with the DNA construct *Amy-GUS* (Fig. 5). Cobombardment with the DNA construct *Act1-Esi47* did not affect the basal level activity of the α-amylase gene promoter. However, *Act1-Esi47* caused the GA induction of the gene promoter to be reduced to only 16-fold. That was a significant and dramatic 77% decrease of the GUS activity produced by the α-amylase gene promoter in GA-treated aleurone compared with the assay without *Act1-Esi47* (Fig. 5). Such inhibition by the *Esi47* gene on the GA induction of the barley α-amylase gene promoter could be partially relieved when the K124Q mutant form of the *Esi47* gene replaced the wild-type *Esi47* in the assays. In the presence of the construct *Act1-Esi47*^{K124Q} the GA induction of the α-amylase gene promoter was 36-fold. *Act1-Esi47*^{K124Q} caused a 48% decrease of the GUS activity controlled by the α-amylase gene promoter compared with the assay without *Act1-Esi47*^{K124Q} in the GA-treated barley aleurone (Fig. 5). These data indicate that the *Esi47* gene inhibits the GA induction of the α-amylase gene in barley aleurone.

The 5′-UTR Mediates the Repression and ABA-Induction of the *Esi47* Gene

The 5′-UTR intron and uORF of the *Esi47* gene were tested for potential roles in mediating regulation of *Esi47* expression. Maize callus tissue was used as host for transient expression assays through biolistic DNA delivery. The 3-kb *Esi47* genomic DNA fragment immediately upstream of the protein kinase ORF start codon was fused with the 5′-UTR intron

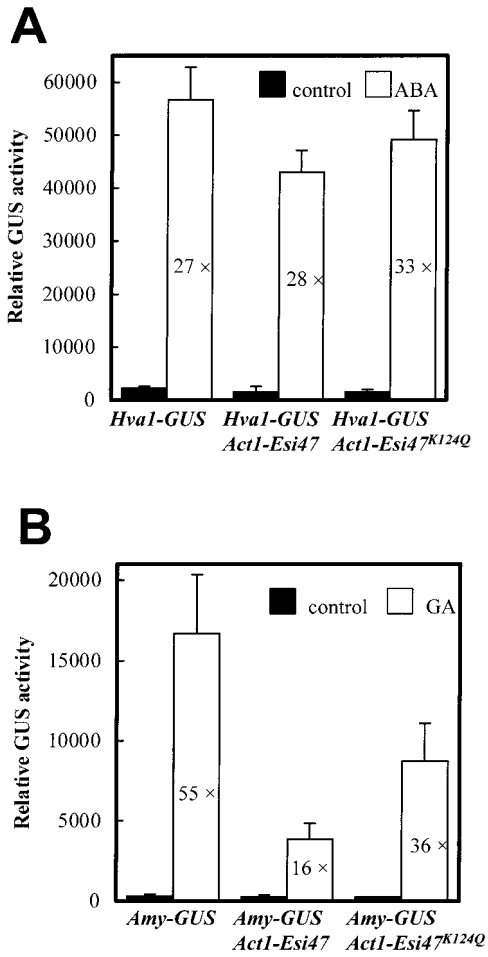

Figure 5. Influence of *Esi47* on the promoter activities of the plant hormone-inducible genes in barley aleurone. Barley aleurone layers were bombarded with the gene constructs indicated. The effector constructs were *Act1-Esi47* (the *Esi47* protein kinase-coding region controlled by the promoter and 5′-UTR intron of the rice actin-1 gene *Act1*) and *Act1-Esi47*^{K124Q} (same as *Act1-Esi47*, but *Esi47* contains the K124Q mutation). A, The barley ABA-inducible *Hva1* gene promoter was fused to the reporter gene for GUS to form the reporter construct *Hva1-GUS* and the bombarded tissues were treated with 20 μM ABA for 24 h as indicated. B, The barley GA-inducible α-amylase gene promoter was fused to the reporter gene for GUS to form the reporter construct *Amy-GUS* and the bombarded tissues were treated with 1 μM GA for 24 h as indicated. In both experiments the controls were not treated with ABA or GA. In all assays GUS activities were measured and normalized against the luciferase activities cobombarded into the barley aleurone. The values are averages of four independent shootings and the SDs are shown as error bars. The induction rates by ABA or GA are also shown.

from the rice actin-1 gene, *Act1*, and the reporter gene for GUS (*Esi47-Act1-GUS*). Modifications of the *Esi47* sequence included the removal of the 5′-UTR intron (*Esi47*$^{\Delta I-1}$*-Act1-GUS*); the corruption of the uORF by removing the A and T nucleotides of the ATG start codon (*Esi47*$^{\Delta uORF}$*-Act1-GUS*); the removal of the 5′-UTR intron and the corruption of the uORF ATG codon (*Esi47*$^{\Delta I-1,\ \Delta uORF}$*-Act1-GUS*). The 5′-UTR intron of the rice *Act1* gene was used to increase the basal level of gene expression (McElroy et al., 1990). The unmodified upstream sequence of *Esi47* caused a 2.6-fold increase of the GUS activity in response to the treatment of 20 μM ABA for 48 h (Fig. 6). The 5′-UTR intron of *Esi47* showed no effects on the GUS activities in control and ABA-treated maize calli (Fig. 6). However, disruption of the *Esi47* uORF increased the basal level of the GUS activity also by 2.6-fold (Fig. 6). Moreover, disruption of the uORF resulted in the loss of the ABA inducibility of the *Esi47* gene (Fig. 6). Therefore, the uORF or the DNA sequence around its ATG codon mediates the repression of the basal level of the *Esi47* gene expression and ABA could relieve such repression. The influence of the uORF on GUS expression was observed in comparisons of constructs *Esi47-Act1-GUS* with *Esi47*$^{\Delta uORF}$*-Act1-GUS* and in *Esi47*$^{\Delta I-1}$*-Act1-GUS* with *Esi47*$^{\Delta I-1,\ \Delta uORF}$*-Act1-GUS*; that is, in the presence and absence of the 5′-UTR intron of *Esi47* (Fig. 6).

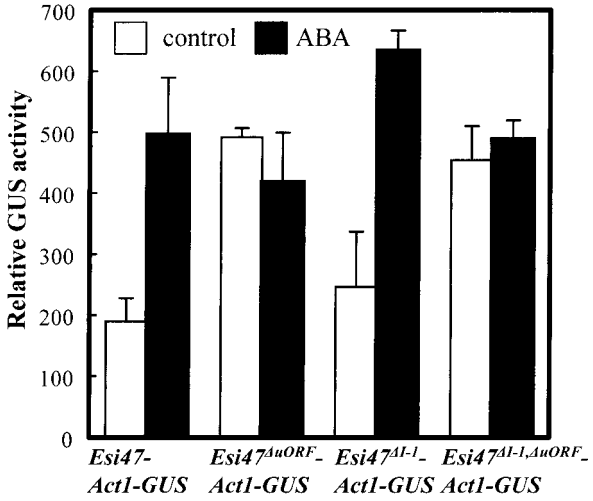

Figure 6. Involvement of the elements in the 5′-UTR of *Esi47* in the regulation of its expression. Maize callus tissues were bombarded with DNA constructs as indicated. *Esi47-Act1-GUS* is the fusion of the 3-kb *Esi47* fragment immediately upstream of the translation initiation of the protein kinase ORF with the 5′-UTR intron from the rice actin-1 gene *Act1* and the reporter gene for GUS. *Esi47*$^{\Delta I-1}$*-Act1-GUS* is the same as *Esi47-Act1-GUS* except the 5′-UTR of Esi47 has been removed. In *Esi47*$^{\Delta uORF}$*-Act1-GUS* the ATG codon of the uORF of *Esi47* has been corrupted, and in *Esi47*$^{\Delta I-1,\ \Delta uORF}$*-Act1-GUS* both the 5′-UTR intron has been removed and the uORF has been corrupted. After DNA delivery the callus tissues were treated with 20 μM ABA for 48 h as indicated; tissues for control were not treated. GUS activities were measured and normalized against the luciferase activities cobombarded to the callus. The values are averages of four independent shootings except for the construct *Esi47-Act1-GUS* with which eight shootings were carried out. The SDs are shown as error bars.

The Arabidopsis Homologs of *Esi47* Are Differentially Regulated by NaCl and ABA

The gene sequences with the highest similarity to *ESI47* that are currently available in the GenBank non-redundant database are from Arabidopsis. In the sequence similarity tree shown in Figure 2 *Esi47* clusters with *F8A24.12*, *F12E4.50*, and *T7F6.28* in a minimal clade. *F8A24.12*, *F12E4.50*, and *T7F6.28* are located in the Arabidopsis chromosomes 3, 5, and 2, respectively. *F8A24.12* and *F12E4.50* are derived from a duplication of segments in chromosomes 3 and 5, as indicated by the profound similarity of the bacteria artificial chromosome clone (F8A24 and F12E4 or MOK16, respectively) sequences encompassing the two genes (Blanc et al., 2000). Because the genome sequence of Arabidopsis has been completely determined, it can be concluded that these three genes are the *Esi47* homologs in Arabidopsis. Coding sequence prediction indicates that all the three genes contain three introns in their coding regions at exactly the same positions as in the *Esi47* gene (Fig. 4). Expressed sequence tags (ESTs) exist in the GenBank database for these Arabidopsis protein kinase genes. One of the ESTs for *F8A24.12* (GenBank accession no. AI993614, derived from the clone 701496785), includes the 5′-UTR of the gene. Comparing the 5′-UTR sequence with the corresponding genomic sequence of the *F8A24.12* gene reveals that this gene, like *Esi47*, also has a small uORF and a 5′-UTR intron that separates the uORF and the protein kinase ORF (Figs. 3 and 4). The length of the *F8A24.12* uORF (27 bp), its sequence, and its distance to the kinase ORF in the mRNA (125 nucleotides) are different from those of the *Esi47* gene (Fig. 3). The 338-bp 5′-UTR intron of *F8A24.12* does not show any sequence similarity to that of the *Esi47* gene (Fig. 3). However, the order of these elements is conserved between the *L. elongatum* gene *Esi47* and the Arabidopsis gene *F8A24.12*. The EST clones available for *F12E4.50* and *T7F6.28* are partial length cDNAs and are too short to determine the presence of any uORFs or introns in their 5′-UTRs (Fig. 4).

Regulation of the expression of these Arabidopsis *Esi47* homologous genes was analyzed by northern-blot hybridization with RNA samples from hydroponically grown Arabidopsis plants. In leaves the expression of the three genes was very weak when the plants were not treated, and only *F8A24.12* was induced by the treatment of 250 mM NaCl (Fig. 7). In roots, all three genes were slightly expressed in control plants and the gene expression was differentially regulated by NaCl and ABA. *F8A24.12* was induced by treatment of 250 mM NaCl within 6 h, but not by treatment of 100 μM ABA in roots (Fig. 7). Though

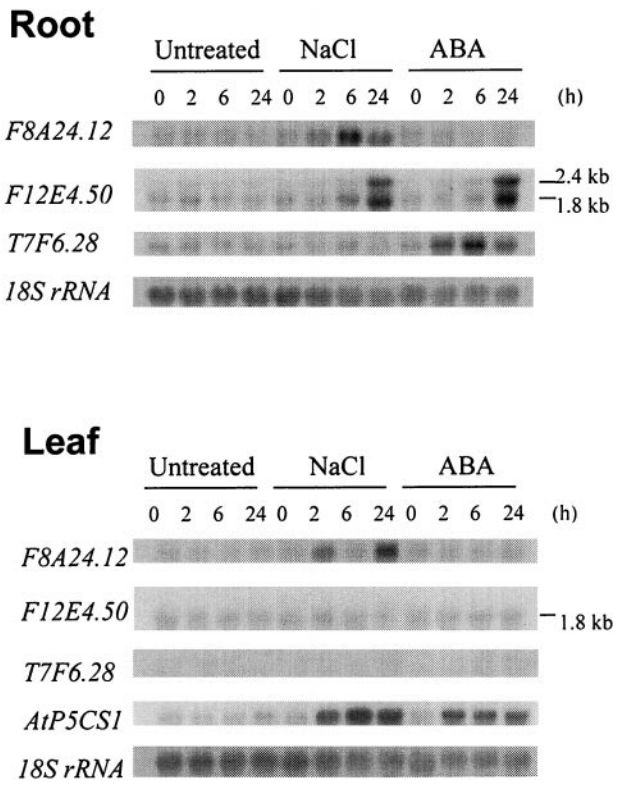

Figure 7. Northern-blot analysis of the expression of the Arabidopsis genes homologous to *Esi47*. Total RNA samples from roots or leaves of hydroponically grown Arabidopsis plants were analyzed. The plants were untreated or treated with 250 mM NaCl or 100 μM ABA for various durations, as indicated. The cDNA inserts from the EST clones for the Arabidopsis genes as indicated were isolated and labeled with ^{32}P as probes. The EST clones for the genes *F8A24.12*, *F12E4.50*, *T7F6.28*, *AtP5CS1*, and *18S rRNA* are F3A9T7, 311H3T7, 193H7T7, FAFM64, and 40F8T7, respectively. The control probe, *AtP5CS1*, was used as an example of a previously characterized NaCl- and ABA-inducible gene.

F8A24.12 was induced in roots and shoots by NaCl treatment, the patterns of expression were different in the two tissues. Induction in roots was high after 6 h of treatment, but had declined by 24 h, whereas the pattern of expression in the leaves was biphasic with gene induction apparent at 2 and 24 h, but not at 6 h. The reliability of the latter result was confirmed by the control probe, *AtP5CS1* for Δ^1-pyrroline-5-carboxylate synthetase (Yoshiba et al., 1995), a salt stress-inducible gene that showed a normal linear pattern of induction when used on the same blot (Fig. 7). In contrast, *T7F6.28* was not induced in roots by NaCl treatment, but could be induced by ABA treatment as early as 2 h (Fig. 7). For *F12E4.50* two transcript bands of 1.8 and 2.4 kb were detected (Fig. 7). This is not likely due to cross-hybridization with a transcript from a related gene since the probe was derived from the EST clone 311H3T7 that corresponds to the 400-bp at the 3′ end of the transcribed sequence of *F12E4.50* and has only 61% sequence similarity with the other Arabidopsis homologues. The levels of the two transcripts were not elevated within 6 h after the onset of any treatments, but they showed elevated levels 24 h after the start of NaCl and ABA treatments (Fig. 7). The fact that the three genes did not show overlapping expression patterns and that these genes are the most related members in a cluster in the phylogenetic tree based on the completed sequence of the Arabidopsis genome (Fig. 2) indicates that the expression patterns revealed by the northern analysis are specific to each gene.

DISCUSSION

Esi47 Is a Plant NAK Group Protein Kinase Gene

By comparing the deduced amino acid sequence of the *Esi47* gene product with those in GenBank and other databases, it is apparent that *Esi47* belongs to the protein kinase gene family since it shows very high scores of similarity to many plant protein kinase sequences. Hardie (1999) constructed a phylogenetic tree of 89 Arabidopsis protein kinases based on the comparisons of the amino acid sequences of the catalytic domains of the kinases. The grouping from the tree agreed well with the biochemical and cellular properties of the kinases. In this study a phylogenetic tree was also built with the amino acid sequences of the catalytic domains of Esi47 and its similar kinases (Fig. 2). This tree shows that the protein kinases that are most similar to Esi47 all belong to the NAK subfamily of plant protein kinases. This group was designated after the Arabidopsis gene *NAK*, which is the first gene identified in this group (Moran and Walker, 1993) and which was named for its novelty, but for which no function or characteristic expression pattern has been described. Like Esi47, the NAK group protein kinases all have a central catalytic domain flanked by short non-catalytic amino and carboxyl domains. Esi47 clusters with four Arabidopsis kinases, F8A24.12, F12E4.50, T7F6.28, and T9I4.2, which form a distinct clade. These Arabidopsis kinases are predicted from the genome sequence, and EST clones have been identified for three of them, F8A24.12, F12E4.50, and T7F6.28. Predictions on intron splicing sites revealed that the four genes all have three introns in the coding regions at exactly the same positions as the introns determined for *Esi47* (Fig. 4). The exon splice junctions of *T7F6.28* were confirmed by sequencing the cDNA insert of the EST clone 193H7T7 (data not shown). In contrast, the protein kinase genes in the adjacent clade that includes *APK1* and *NAK* in the phylogenetic tree have four introns in the coding regions, all at different positions from those of *Esi47* (not shown).

None of the protein kinase genes in the NAK group has been investigated for roles in signaling, except for the Arabidopsis gene *ARSK1*, which was only shown to be up-regulated in roots by salt stress or ABA treatment (Hwang and Goodman, 1995). This study demonstrates cellular function for a member of

this group of plant protein kinase genes by showing that *Esi47* is involved in the ABA-promoted suppression of GA action in barley aleurone.

Esi47 Is Involved in Plant Hormone Signaling

Protein kinases play key roles in detecting and relaying developmental and environmental signals for the regulation of specific genes and thus mediate cellular responses to those signals. In this study we transiently expressed *Esi47* in barley aleurone to assess its roles in plant hormone signaling. Species in the Gramineae, including barley, wheat, and *L. elongatum*, possess aleurone layer tissues in their seeds. During seed germination, aleurone cells synthesize and secrete a variety of hydrolytic enzymes for breaking down and mobilizing nutrients stored in endosperm starch, protein, and lipid. During germination GA in aleurone cells promotes the production of the hydrolytic enzymes by up-regulating the genes for these enzymes, whereas ABA prevents germination in part by inhibiting the GA action in the aleurone tissues. The ease of obtaining the tissues and the availability of a number of well-characterized ABA- or GA-regulated genes from these tissues made the barley aleurone a model system for studying the ABA and GA signaling in plants (Lovegrove and Hooley, 2000). Since *Esi47* can be induced by ABA in *L. elongatum* roots (Galvez et al., 1993), it may mediate certain cellular processes related to ABA and its antagonist GA in aleurone cells.

Our results showed that *Esi47* had no effects on the basal level expression of the ABA-inducible *Hva1* gene promoter (Fig. 5). There was no significant effect on the degree of induction of the promoter by ABA. Though the 24% inhibition by *Esi47* on the absolute level of GUS expression driven by the *Hva1* gene promoter in aleurone tissues treated with ABA was slight, it was nevertheless statistically significant ($P = 0.014$, Fig. 5). However, there is no significant difference between the effects of the *Esi47* gene and its K124Q mutant form on the *Hva1* promoter in the ABA-treated aleurone tissues. There is also no difference between the assays with *Act1-Esi47*^{K124Q} and the assays without effector DNA (Fig. 5). Therefore, the relationship between *Esi47* and the ABA induction of *Hva1* needs to be investigated in another independent way.

Esi47 also had no influence on the activity of the low-pI α-amylase gene promoter when the tissues were not treated with GA, but it substantially reduced the GA induction of the α-amylase gene promoter (Fig. 5). Moreover, such inhibition of GA action might be partially dependent on the protein kinase activity of the *Esi47* gene product since the K124Q mutant form of the *Esi47* gene had a much less degree of inhibition on the GA induction of the α-amylase gene promoter (Fig. 5). Thus, the effects of *Esi47* in barley aleurone are very similar to those of the wheat gene *PKABA1*, which encodes an SNF1-related protein kinase (Fig. 2). *PKABA1* had been shown to inhibit the GA induction of a number of barley genes for hydrolytic enzymes, including low- and high-pI α-amylases and a Cys proteinase (Gómez-Cadenas et al., 1999). Like *Esi47*, *PKABA1* is induced by ABA, salt, and water deficit stresses (Anderberg and Walker-Simmons, 1992; Holappa and Walker-Simmons, 1995). Thus, the two kinase genes likely participate in the same cascade of signaling pathway or in two independent pathways that inhibit the GA induction of the expression of genes for hydrolytic enzymes. The transcripts for the *PKABA1*-like gene in barley aleurone could be detected and the levels could be elevated by ABA treatment. *Esi47* is only known to be induced by ABA or stress in vegetative tissues (Galvez et al., 1993). In this study confirmation on whether the barley homologous gene of *Esi47* is induced by ABA in aleurone was not attempted. Since the *Esi47* gene showed effects on gene expression in aleurone, it could be assumed that at least the pathway downstream of the *Esi47*-like gene is present in seed aleurone and is susceptible to activation. Therefore, *Esi47* and *PKABA1* could mimic ABA in suppressing the GA induction of the genes for hydrolytic enzymes in barley aleurone (Fig. 8).

Phospholipase D (PLD) has been shown to mediate the ABA-promoted suppression of GA action in aleurone tissues. Exogenous ABA could promptly induce the PLD activity and the increase of the PLD activity product phosphatidic acid in barley aleurone protoplasts (Ritchie and Gilroy, 1998). Treatment of aleurone protoplasts with phosphatidic acid not only caused inhibition of the GA-promoted production of α-amylase, but enhanced the production of ABA-induced gene products amylase subtilisin inhibitor

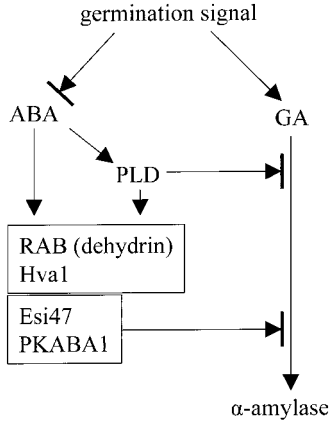

Figure 8. A schematic diagram of the involvement of the *Esi47* gene in stress and hormone signaling. ABA increases the PLD activity, which in turn suppresses the GA induction of the genes for hydrolytic enzymes including α-amylase. ABA induces the expression of *Esi47*, *PKABA1*, *RAB*, and *Hva1* in a PLD-dependent or -independent fashion. *Esi47* and *PKABA1* suppress the GA induction of the α-amylase gene depending on or independently of PLD activity.

and the dehydrin proteins, mimicking the actions of ABA. The rapid induction of the PLD activity by ABA indicates that PLD might be an early component in the ABA signaling pathway, leading to the suppression of GA action and may act upstream of *Esi47* and *PKABA1* (Fig. 8).

Such a model may be used as guide for further investigation on the roles of *Esi47* and *PKABA1* in stress and ABA responses in vegetative tissues. Although the genes may perform different cellular functions in vegetative tissues from those in aleurone, segments of the signaling pathways may be well conserved. Because GA promotes cell growth, it is an intriguing possibility that activation of *Esi47* or *PKABA1* may cause transient cell growth arrest by suppressing the GA-mediated functions upon salt and water deficit stresses so as to allow cells to adjust to the new, unfavorable environmental conditions.

Regulation of the Expression of *Esi47* and Its Arabidopsis Homologs

Esi47 is up-regulated by salt and ABA in roots of *L. elongatum* and wheat (Gulick and Dvořák, 1992; Galvez et al., 1993). The presence of an intron and the more unusual small uORF in the 5′-UTR of the gene suggests that these might play a role in the regulation of gene expression. It is interesting to observe that the structure of one of the *Esi47*-homologous genes in Arabidopsis, *F8A24.12*, is the same as that of *Esi47* (Figs. 3 and 4). Our analysis of gene expression in maize callus showed that the DNA sequence around the ATG start codon of the uORF mediates the repression of the basal level expression of *Esi47* and such repression may be relieved by ABA (Fig. 6). This experiment did not determine if the uORF sequence-mediated gene repression was at the level of transcription or translation. This might be solved by comparing the transcript levels of the *GUS* reporter gene with the measured GUS activities, which represent the gene expression at translational level.

It is possible that the RNA sequence surrounding and including the AUG start codon for the uORF represents a repressor element, for example, forming a secondary structure that prevents the moving of the ribosomal subunit. A more plausible explanation is that translation of the uORF mediates the repression of the gene and the modification of the AUG codon of the uORF alleviated such repression. Upstream ORFs occur in about 7% to 10% of the plant genes and have been implicated in reducing the translation efficiency of the downstream major ORFs, as demonstrated by the maize *Lc* gene uORF (Damiani and Wessler, 1993; Fütterer and Hohn, 1996; Gallie, 1996). In the scanning model for translation of plant mRNAs the ribosome subunit binds to the 5′ end of mRNA, moves downstream, and initiates translation once it encounters the first AUG codon. The reinitiation at the AUG codon for the downstream major ORF is partially or completely inhibited with the presence of uORF, and consequently, the translation of the major ORF is repressed. Reinitiation in the yeast *GCN4* gene, which contains four uORFs, is dependent on the degree of phosphorylation of the translation factor eIF2α, and such phosphorylation is promoted by amino acid deprivation in culture medium (Hinnebusch, 1996). Such translational control by environmental conditions may suggest a mechanism for the probable uORF-mediated repression of the *Esi47* gene since it is also regulated by environmental stresses.

Different species may have similar genes, but their unique characteristics may be due to the unique regulation of gene expression. Although the *L. elongatum* *Esi47* gene is inducible by salt stress and ABA in roots, its three Arabidopsis homologous genes have evolved specific salt and ABA regulation patterns. *F8A24.12* can be induced by NaCl, but not by ABA, whereas *T7F6.28* is ABA inducible, but not NaCl inducible (Fig. 7). Only *F12E4.50* is up-regulated by NaCl and ABA, but the increase of *F12E4.50* transcripts is much slower than those of *F8A24.12* and *T7F6.28* (Fig. 7). In contrast, the expression of *F8A24.12* in leaves is induced by NaCl, but not ABA; neither of the other homologs is up-regulated in leaves in response to either stress. It is likely that the ancestral *Esi47*-like gene that existed before the separation of monocot and dicot plants was regulated by salt stress and ABA in roots. The regulation patterns of the three Arabidopsis *Esi47* homologs indicate the separation of the salt and ABA signaling pathways in dicot plants. Such separation was facilitated by the gene duplication that gave rise to *F8A24.12* and *F12E4.50*. It is not clear how the *T7F6.28* gene evolved. It is also interesting that there are two transcripts with different sizes detected for *F12E4.50* in Arabidopsis roots, but not in leaves (Fig. 7). Therefore, a mechanism of alternative transcription or RNA processing might exist for *F12E4.50* as an additional level of gene regulation. Moreover, the biphasic pattern of salt induction of *F8A24.12* in leaf tissue suggests that it is regulated by multiple signaling pathways.

The study of the regulation of the Arabidopsis *Esi47* homologs by stress and ABA would advance the investigation of the mechanisms of regulation of the *Esi47*-like genes. For example, since the Esi47 uORF was shown to mediate gene repression and ABA regulation, the similarity in the 5′-UTR structure of *Esi47* and *F8A24.12* suggests that such a mechanism may be well conserved in the *Esi47*-like genes. Further investigation of the regulation of the Arabidopsis *Esi47* homologs and the comparison with that of the *Esi47* gene and its possible emerging paralogs in grass species would result in much information not only about the mechanisms of gene response to environmental stresses and plant hormones, but also about the evolution of gene regulation. The partial

sequence of an EST clone from bread wheat that shows 94% nucleotide sequence identity to *Esi47*, and is the apparent homolog of *Esi47*, has recently been deposited in GenBank's EST database (accession no. BE518403). The accumulation of further DNA sequences in the ongoing genomic programs in the grass species is likely to significantly expand our current view of this protein kinase gene family in monocot species.

MATERIALS AND METHODS

Plant Materials

Lophopyrum elongatum (Host) A. Love plants were grown hydroponically in greenhouse as previously described (Gulick and Dvořák, 1987) and were treated with 250 mM NaCl for 6 h before the roots were collected.

Arabidopsis Columbia ecotype strain Col-0 used in this study was obtained from the Arabidopsis Biological Resource Center, Ohio State University (Columbus, OH). Arabidopsis plants were grown hydroponically according to Gibeaut et al. (1997) under light intensity of 5,000 lux at 22°C. The photoperiod for the first 5 weeks of growth was 8 h of light and 16 h of darkness, and plants were then switched to constant light for 2 d before treatment with 250 mM NaCl or 100 μM ABA. Plants were treated with NaCl or ABA for 2, 6, and 24 h, and control plants were untreated.

RNA Extraction

Plant materials were ground in liquid nitrogen and suspended in 0.2 M sodium borate, 30 mM EGTA, 2% (w/v) SDS, and 1% (w/v) sodium lauroyl sarcosine, pH 9.0. Samples were extracted with phenol:chloroform (1:1, v/v) and then with chloroform. RNA was precipitated with 2 M LiCl. The RNA-containing pellets were dissolved in water and reprecipitated with one-tenth volume of 3 M sodium acetate, pH 6.0 and 2 volumes of ethanol and were then dissolved in water. Poly(A)-RNA was purified with poly(U)-Sephadex (Gibco-BRL, Gaithersburg, MD).

Library Construction and Screening

RNA samples from roots of *L. elongatum* treated with 250 mM NaCl for 6 h were used for cDNA library construction with the λ Zap II vector (Stratagene, La Jolla, CA) according to the protocol of the manufacturer. Inserts were ligated to the vector at the *Eco*RI site for 5′ ends of cDNAs and the *Xho*I site for 3′ ends. After screening the library with the ^{32}P-labeled probe derived from the original partial *Esi47* cDNA (Gulick and Dvořák, 1992), cDNAs from the positive clones were rescued in the pBluescript SK$^-$ plasmid vector by the in vivo excision method according to the protocol of the manufacturer.

L. elongatum leaf genomic DNA was partially digested with *Sau*3A, such that most of the DNA fragments were in the range of 10 to 20 kb, and was used in library construction with the λ Fix II vector (Stratagene) according to the procedures recommended by the manufacturer. The recombinant DNA was packaged with the Gigapack Gold packaging extracts (Stratagene). The *Esi47* cDNA was used as ^{32}P-labeled probe to isolate clones for *Esi47*. Positive clones were purified and subcloned in the plasmid vector pBluescript SK$^-$. DNA fragments corresponding to the *Esi47* gene were identified by Southern analysis. Two overlapping subclones contain, respectively, a 3.3-kb *Xho*I fragment for the 3-kb of DNA upstream of the coding region and part of the coding region, and a 4.3-kb *Kpn*I fragment for the 3′-flanking region and most of the transcribed region.

Plasmid Construction

The *Pfu* DNA polymerase (Stratagene) was used for all PCR. The *Escherichia coli* strain XL-1 Blue (Stratagene) was used as host for the plasmids.

To obtain the K124Q mutant form of the *Esi47* gene product, PCR was carried out with cDNA clone. An A to C change in the codon for Lys-124 was included in the primer 5′-GCACCCTCGAGCCGCGCCGAAGCCTCGATGTCGCCATCCAGCAGCTCG-3′, which contains an *Xho*I site. The antisense primer, 5′-CTCACAAGCTTGGGTGTC-3′, is located close to the translation termination site and included a *Hin*dIII site. The PCR fragment was digested with *Xho*I and *Hin*dIII and was used to replace the corresponding fragment of the wild-type cDNA.

For transient expression in barley aleurone, the *Esi47* protein kinase ORF or its K124Q mutant form was PCR amplified from the cDNA with the sense primer 5′-AGCAGAAGATCTATGCAGTGCTTC-3′ (with a *Bgl*II site) and the antisense primer 5′-TCCATATCTAGAGTCATTGTGTTC-3′ (with an *Xba*I site). The PCR fragments were digested with *Bgl*II and the end was made blunt by filling with the Klenow enzyme, and was subsequently digested with *Xba*I. Such DNA fragments were ligated to the *Sma*I- and *Xba*I-digested vector pCOR113, which contains the promoter and the 5′-UTR intron of the rice actin-1 gene *Act1* (McElroy et al., 1991). The resulting plasmid constructs are designated Act1-Esi47 and Act1-Esi47^{K124Q}, respectively.

For transient expression in maize callus, the 3-kb *Esi47* genomic DNA immediately upstream of the protein kinase-coding region was amplified by PCR from the *Esi47* genomic subclone. The sense primer was the M13 reverse primer 5′-AGCGGATAACAATTTCACACAGG-3′, corresponding to a region close to the cloning sites in the vector pBluescript SK$^-$; the antisense primer was 5′-GAAGCTCTAGAATCGCGCTTCTGCTTTG-3′, which contains an *Xba*I site. The PCR fragment was digested with *Xba*I and *Xho*I (the latter is contributed by the multiple cloning sites of pBluescript SK$^-$) and ligated to the vector pDMC205 (McElroy et al., 1995) digested with the same pair of enzymes. The *Esi47* sequence was thus placed upstream of the rice *Act1* gene 5′-UTR intron and the reporter gene for GUS from the vector and formed the construct Esi47-Act1-GUS. To corrupt the ATG codon for the *Esi47* uORF, the genomic subclone was digested with *Nco*I

whose site overlaps with the ATG codon. The cohesive ends were then filled with dCTP, made blunt by treatment with mung bean nuclease, and circularized by T4 DNA ligase. Such a modified *Esi47* upstream sequence was cloned in pDMC205 in the same way for Esi47-Act1-GUS to form Esi47$^{\Delta uORF}$-Act1-GUS. To remove the *Esi47* 5′-UTR intron, the 134-bp *Nco*I-*Pst*I cDNA fragment that encompasses the splicing site of the intron was used to replace the corresponding genomic sequence in the genomic subclone. The *Esi47* genomic DNA without the 5′-UTR intron was cloned in pDMC205 to form Esi47$^{\Delta I-1}$-Act1-GUS. The *Esi47* genomic sequence without the 5′-UTR intron was also modified to corrupt the ATG codon of the uORF inthe same way as mentioned above and the resulting genomic DNA was cloned in pDMC205 to form Esi47$^{\Delta I-1,\ \Delta uORF}$-Act1-GUS.

Northern-Blot Analysis

Northern-blot hybridization was carried out according to Sambrook et al. (1989). Twenty micrograms of total RNA was separated in 1% (w/v) denaturing agarose gels containing 6% (w/v) formaldehyde and 1× MOPS [3-(*N*-morpholino)-propanesulfonic acid] buffer. RNAs were then transblotted to the Hybond-N nylon filters (Amersham, Buckinghamshire, UK) by capillary transfer using paper towels. cDNA probes were ^{32}P-labeled with random primers (Amersham) and used for hybridization with RNA blots in 50% (v/v) formamide, 5× SSC, 5× Denhardt's solution, 0.5% (w/v) SDS, and 0.1 mg mL^{-1} denatured herring sperm DNA at 42°C for 20 h. The blots were washed in 1× SSC, 0.1% (w/v) SDS at 50°C and x-ray films were exposed for various time periods with intensifying screens at −80°C. The EST clones used in this study were obtained from the Arabidopsis Biological Resource Center. They were F3A9T7 (*F8A24.12*), 311H7T7 (*F12E4.50*), 193H7T7 (*T7F6.28*), FAFM64 (*AtP5CS1*), and 40F8T7 (*18S rRNA*). The probe for the *18S rRNA* gene was included for demonstration of equal loading of RNA samples.

Primer Extension

Ten picomoles of an antisense primer 5′-ACAACCGACCAACCAACTGCTC-3′ was end-labeled in a 10-μL mixture containing 50 μCi [γ-^{32}P]ATP (3,000 Ci mmol^{-1}, ICN, Irvine, CA) and 6 units of T4 DNA kinase at 37°C for 30 min. Approximately 5 × 10^6 cpm of the labeled primer was used in the reverse transcription of 1.5 μg of poly(A)-RNA derived from *L. elongatum* roots treated with 250 mM NaCl for 6 h. The primer and RNA were mixed with 1.5 μL of the 5× reaction buffer (250 mM Tris-HCl, pH 8.3, 250 mM KCl, 20 mM $MgCl_2$, and 50 mM dithiothreitol) in a volume of 13 μL. The mixture was heated at 68°C for 2 min and let to cool slowly to 37°C. Then dATP, dCTP, dGTP, and dTTP were added to 1 mM each, along with 5 units of Moloney murine leukemia virus reverse transcriptase (Pharmacia Biotech, Piscataway, NJ) to make a final volume of 15 μL. The reaction was carried out at 37°C for 1 h and was stopped by heating at 65°C for 10 min. Four microliters of the sample was run on a 6% (w/v) polyacrylamide sequencing gel.

Transient Gene Expression

The procedures for DNA bombardment on barley aleurone were carried out essentially according to Gómez-Cadenas et al. (1999). The effector plasmids were Act1-Esi47 and Act1-Esi47^{K124Q}. Two reporter plasmids were used; one was Hva1-GUS (QS264; Straub et al., 1994) in which the promoter of the barley ABA-induced *Hva1* gene was fused to the reporter gene for GUS and the other was Amy-GUS (MBL022; Lanahan et al., 1992) in which the promoter of the barley GA-induced low-pI α-amylase gene *Amy32b* was fused to GUS. Plasmid pAHC18 (Bruce et al., 1989), which contains the ubiquitin gene promoter-luciferase construct, was used as an internal control to measure the DNA delivery efficiency. Each bombardment included an effector plasmid, a reporter plasmid, and pAHC18; or as controls, only a reporter plasmid and pAHC18 were used in each bombardment. Embryoless Himalaya barley half-seeds were used as target tissue. After each bombardment, one-half of the half-seeds were treated with 20 μM ABA (in experiments with the Hva1-GUS reporter plasmid) or 1 μM GA (in experiments with the Amy-GUS reporter plasmid) for 24 h. The rest of the half-seeds were not treated and were used as control. The GUS and luciferase activities of the homogenized half-seeds were measured. The GUS activities were normalized against the luciferase activities of the corresponding samples. Four independent bombardments were done for each combination of the plasmid constructs.

Maize embryogenic callus tissue was used for expressing the DNA constructs Esi47-Act1-GUS, Esi47$^{\Delta uORF}$-Act1-GUS, Esi47$^{\Delta I-1}$-Act1-GUS, and Esi47$^{\Delta I-1,\ \Delta uORF}$-Act1-GUS by particle bombardment. The callus tissue was maintained in the $AgNO_3$-free callus maintenance medium for 2 weeks before bombardment. Plasmid DNA concentration used for shooting was adjusted to 1 μg μL^{-1} and each time 2.5 μg of each DNA construct was used to coat gold particles. Plasmid pJD312 in which the gene for luciferase is controlled by the cauliflower mosaic virus *35S* promoter (Luehrsen et al., 1992) was included in each bombardment as a control to assay the efficiency of DNA delivery. Immediately after bombardment, callus tissue was placed in the callus maintenance medium containing 20 μM ABA and was incubated at 25°C in the dark for 48 h. The control samples were incubated in the same way, but ABA was not added to the medium. The tissues were then homogenized and the GUS and luciferase activities of tissue extracts were measured as mentioned above. The statistical significance of the data was determined by the Student's *t* test.

Construction of the *Esi47* Phylogenetic Tree

The full-length amino acid sequence of the Esi47 protein kinase was used in a BLASTP search for its most similar protein kinases in the databases. The catalytic domain of Esi47 was determined according to Hanks et al. (1988) and

Hanks and Quinn (1991). The catalytic domains of the Esi47-like kinases were then determined by aligning their full-length amino acid sequences with that of Esi47. These catalytic domain sequences were then aligned by the CLUSTAL method (Higgins and Sharp, 1988) with the PC/GENE computer software package (IntelliGenetics, Mountain View, CA). The phylogenetic tree was thus built based on the multiple sequence alignment.

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